

# Bax expression and growth behaviour of basal cell carcinomas.

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# Bax expression and growth behavior of basal cell carcinomas

**Background:** To understand the typical growth behavior of basal cell carcinoma (BCC) we searched for the correlation between proliferation and apoptosis and progression of BCC.

**Methods:** Expression of Bcl-2, Bax, and Ki-67 was immunohistochemically investigated in both normal skin and BCC cells, as well as in the epidermis overlying BCC.

**Results:** The results showed that in normal epidermis, Bcl-2 was homogeneously expressed in the basal cell compartment, whereas Ki-67 expression was largely restricted to the parabasal layer, the layer just above the basal cell layer, and exhibited a more scattered staining pattern. Bax was occasionally expressed in the basal layer and widely in the suprabasal compartment. Strikingly, the apparently normal epidermis overlying BCC showed an increased Bcl-2 staining. In BCC, cells stained homogeneously for Bcl-2, whereas Bax and Ki-67 showed scattered staining patterns. Simultaneous expression was seen for Bcl-2 and Bax in  $80 \pm 7\%$  of the tumor cells, and co-expression of Bcl-2 and Ki-67 in  $20 \pm 7\%$  of the tumor cells. The cells expressing Bcl-2 and Ki-67, but lacking expression of Bax, the progressive fraction, comprised on average  $7 \pm 3\%$  of the tumor cell population.

**Conclusion:** These results suggest that this small progressive fraction of tumor cells, in combination with the relatively high percentage of cells still prone to apoptosis, can explain the indolent growth behavior of BCC.

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Basal cell carcinoma (BCC), the most common malignancy in Caucasians, is a non-melanoma type of skin cancer arising predominantly in the face and neck. It is a typically slowly growing tumor with a great diversity in histopathological appearance.<sup>1</sup> Although generally of indolent nature, occasionally the tumor can become very aggressive and destructive, a clinical behavior that is mainly related to the morpheiform, metatypical or micronodular phenotype.<sup>1</sup>

In general, growth of a tumor is based on a derailment of cell proliferation.<sup>2</sup> However, it has been shown in previous studies that the duration of a cell cycle in BCC resembles that of keratinocytes of the generative compartment of normal epidermis, approximately 217 h.<sup>3</sup> This is in contrast with the clin-

ical observation that BCC is an extremely slowly growing tumor, which may take months or even years to double in size.<sup>4</sup> It has therefore been suggested that only a small percentage of all tumor cells is actively proliferating and it was found that these cells are mainly located at the periphery of the tumor nests.<sup>5,6</sup> In addition, an increase of the apoptotic potential of BCC cells may be another explanation for this phenomenon.

Apoptosis is partly regulated by the balance between the expression of anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax (Bcl-2 associated x-protein). The Bcl-2 gene was first discovered in B-cell-lymphomas showing a t(14:18) translocation.<sup>7</sup> The resulting fusion gene yielded an overexpression of the Bcl-2

protein.<sup>8</sup> This translocation is frequently found in various cancers and it was shown by Reed<sup>9</sup> that the Bcl-2 protein could suppress apoptosis induced by various stimuli. Bax was discovered in an immunoprecipitation assay when it co-precipitated with Bcl-2. Although it exhibits a high degree of homology with Bcl-2, this protein showed pro-apoptotic activity.<sup>10</sup> Overexpression of Bax results in homodimerization and suppression of the functionality of Bcl-2, followed by the induction of apoptosis. Overexpression of Bcl-2, however, results in cell survival. It can thus be concluded that the relative expression levels of these two proteins in part determine the fate of a cell.<sup>10</sup> Since most tumor cells in BCC show expression of Bcl-2,<sup>1</sup> the expression level of the Bax protein may determine whether a cell lives or dies.

The slow expansive growth of BCC must therefore be explained in the light of the delicate balance between cell generation (proliferation) and cell loss (apoptosis), which is responsible for the tissue homeostasis in multicellular organisms, and becomes disturbed in tumor development.

The aim of this study was to determine and quantify the cell population in BCC that is responsible for its expansion, i.e. the tumor cells that are protected from undergoing apoptosis (Bcl-2-positive, Bax-negative) and are actively proliferating (Ki-67-positive). It was found that this cell population comprises a minor fraction of the tumor cells and is smaller than the cell fraction that can potentially undergo apoptosis.

## Materials and methods

### Tissue material

Normal skin was obtained from breast reduction surgery at the Department of Plastic Surgery of our hospital and was snap-frozen in liquid nitrogen. Frozen sections (5 µm) were cut and placed on slides coated with 3-aminopropyltriethoxysilan (APTS) (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands).

Fresh frozen tumor material was obtained from 30 BCC patients treated by Mohs' micrographic surgery. The histological classification of the BCCs showed that 12 were nodular type, three were micronodular type, one was morpheaform type, two were nodular/morpheaform type, six were micronodular/morpheaform type, two were nodular/adenoid type and four were mixed type (Table 3). In all these cases, the apparently normal overlying epidermis was also examined. Frozen sections (5 µm) were cut and placed on APTS-coated slides.

## Methods

### Immunohistochemistry

Specificity of the polyclonal antibodies was warranted by the fact that they are both affinity-purified using

the synthetic peptide used to immunize the rabbits. Furthermore, immunoblotting was performed for the monoclonal and polyclonal antibody against Bcl-2 and the polyclonal antibody against Bax as described previously,<sup>11</sup> using the following cell lines: HeLa (human cervix adenocarcinoma), HT29 (human colorectal adenocarcinoma), 3T3 (mouse fibroblast), MCF-7 (human adenomacarcinoma of the mammary gland), and Jurkat (human T-cell leukemia) (ATCC in Manassas, VA, USA). In addition, the polyclonal antibodies against Bax and Bcl-2 were pre-absorbed with the peptides used for immunization.

### Immunoperoxidase staining

Single antigen detection was performed using the immunoperoxidase staining method.<sup>12</sup> Serial frozen sections were fixed in acetone and endogenous peroxidase activity was quenched by incubating the slides with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS). Non-specific binding was blocked by incubation with normal goat serum. The slides were then incubated overnight at 4°C with the primary antibodies in optimal dilutions (Table 1).

The bound primary antibodies were linked with biotinylated secondary antibodies. The sections were then incubated with avidin-biotin complex horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA, USA). Peroxidase activity was detected with the chromogen 3,3'-diaminobenzidine (DAB) and the sections were counterstained with hematoxylin (Fluka AG, Buchs, Germany).

### Double-immunofluorescence staining

Serial frozen sections were fixed in acetone and non-specific binding was blocked by incubation with normal goat serum. The slides were then incubated with the primary antibodies, either subsequently or simultaneously, overnight at 4°C. Table 2 shows the combinations of antibodies that were used.

For combinations A and B (Table 2) the overnight incubation was followed by incubation with peroxidase-conjugated goat-antimouse IgG (DAKO A/S, Glostrup, Denmark) and subsequently incubation with a mixture of Texas Red-labeled goat-antirabbit Ig (ITK Diagnostics, Uithoorn, the Netherlands) and fluoresceine isothiocyanate (FITC)-labeled tyramide<sup>13</sup> to detect peroxidase activity and simultaneous signal amplification.

For combinations C, D and E (Table 2), the slides were incubated with a mixture of FITC-labeled goat-antimouse Ig and Texas Red-labeled goat-antirabbit Ig.

For combination F, overnight incubation with the polyclonal Bax antibody was followed by incubation with biotinylated goat-antirabbit and avidin-biotin

Table 1. Antibody characteristics and dilutions used in the single label immunoperoxidase staining procedure

Antibody epitope	Manufacturer	Dilution
Monoclonal anti-Bcl-2 (clone 124) aa.41–54	DAKO A/S, Glostrup, Denmark	1:50
Monoclonal anti-Bcl-2 (clone 100) aa.44–52	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	1:25
Polyclonal anti-Bcl-2 (N-19) N-terminal	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	1:750
Polyclonal anti-Bax (P-19) N-terminal	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	1:100
Polyclonal anti-Bax (Ab-1) aa.150–165	Oncogene Research Products, Cambridge, MA, USA	1:20
Monoclonal anti-Ki-67 (MIB-1)	Immunotech, Marseille, France	1:50
Polyclonal anti-Ki-67	DAKO A/S, Glostrup, Denmark	1:200

aa, amino acids.

Table 2. Combinations of primary antibodies applied in the double-immunofluorescence assay

Combination	Antibodies and dilutions
A	MAb Bcl-2 (clone 100) 1:10, PAb Bcl-2 1:200, simultaneous incubation
B	MAB Bcl-2 (clone 124) 1:50, PAb Ki-67 1:200, simultaneous incubation
C	PAb Bcl-2 1:200, MIB-1 1:25, simultaneous incubation
D	MIB-1 1:25, PAb Bax (P-19) 1:200, simultaneous incubation
E	MAB Bcl-2 (clone 124) 1:50, PAb Bax (P-19) 1:200, simultaneous incubation
F	PAb Bax (P-19) 1:1000, PAb Bcl-2 1:200, subsequent incubations

Pab, polyclonal antibody; Mab, monoclonal antibody.

complex (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA, USA). Detection of peroxidase activity and simultaneous signal amplification was achieved by incubation with tetramethylrhodamine isothiocyanate (TRITC)-labeled tyramide.<sup>13</sup> The slides were then incubated with the polyclonal Bcl-2 antibody and subsequently with FITC-labeled goat-antirabbit Ig (ITK Diagnostics, Uithoorn, the Netherlands).

#### Determination of percentages of positive cells

The percentages of positive cells were estimated by counting 500 cells per tumor by two independent observers.

## Results

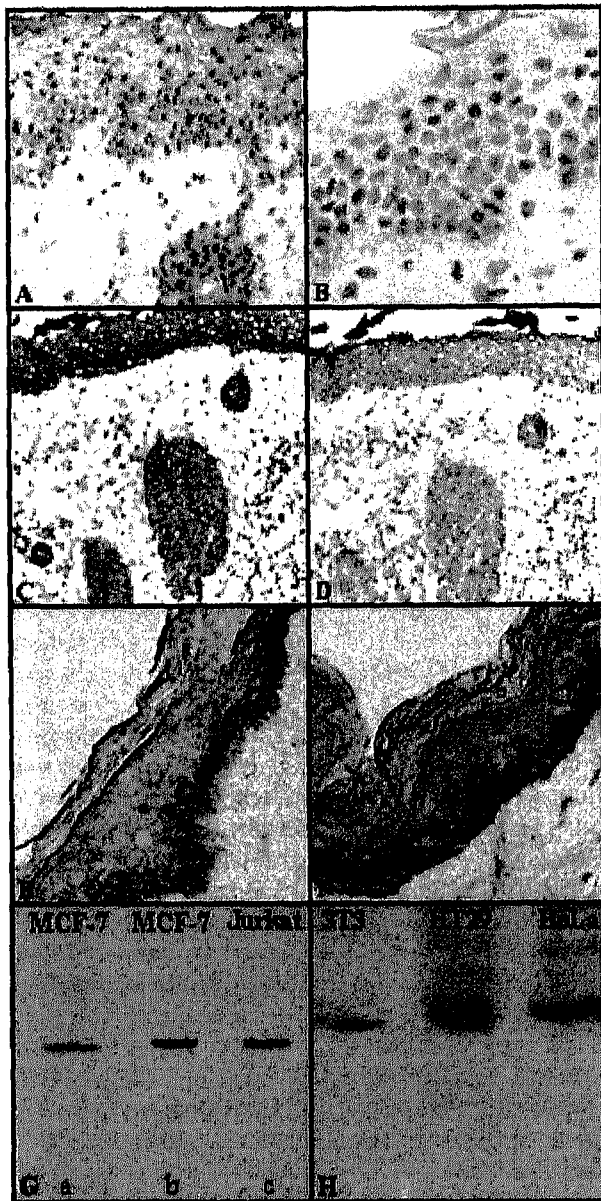
#### Specificity testing of the immunoreagentia

The specificity of the polyclonal and monoclonal antibodies was warranted by several tests. Firstly, the polyclonal antibodies against Bax and Bcl-2 were pre-absorbed with the peptides used for immunization, resulting in absence of staining when the corresponding peptide was used (Fig. 1A–D). Secondly, in normal epidermis, the monoclonal antibody against Bcl-2 showed the same expression pattern of the protein as seen with the polyclonal antibody (Figs. 1E and F, respectively). In addition, the results of the immunoblotting showed the expected 29-kDa band for both the monoclonal and the polyclonal antibody against Bcl-2 (Fig. 1G). Furthermore, the expected 21-kDa band for the polyclonal antibody against Bax was seen (Fig. 1H).

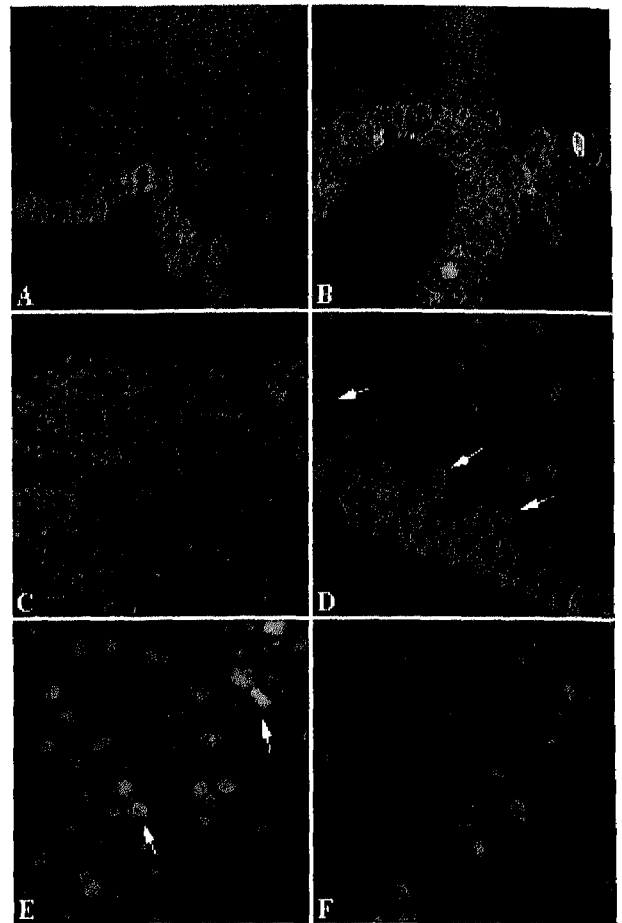
#### Proliferation and apoptosis markers in the epidermis

In normal skin, the expression of the Bcl-2 protein was found to be restricted to the basal cell layer of the epidermis (Fig. 2A). This distribution pattern was seen with the monoclonal as well as the polyclonal antibodies. Ki-67 expression was found to be predominantly restricted to cells present in the parabasal layer, the layer just above the basal cell layer (Fig. 2A). These cells were either scattered throughout this layer, or clustered in certain areas. Again the polyclonal and monoclonal antibodies gave rise to the same results. In general, the staining patterns of Ki-67 and Bcl-2 were mutually exclusive, but occasionally some basal cells showed simultaneous expression. Bax was found to be expressed in the suprabasal layers of the epidermis, and in an occasional basal cell. The double immunofluorescence studies also revealed mutually exclusive expression of Bcl-2 and Bax proteins in normal skin (Fig. 2C). Bax and Ki-67 occasionally showed co-expression in the normal epidermis (Fig. 2E).

We noticed some striking differences in the epidermis overlying BCC as compared with the epidermis of normal skin. Epidermis overlying BCC showed increased numbers of cells positive for Ki-67, with suprabasal layers of the epidermis also showing positive cells. With the monoclonal Bcl-2 antibody, a staining pattern similar to that in normal skin was seen, but when using the polyclonal Bcl-2 antibody, also para- and suprabasal cells showed positive staining. As a result, cells with overlapping expression of Bcl-2 and Ki-67 were shown (Fig. 2B). Also, Bax and Bcl-2 could be detected simultaneously in the suprabasal layers of the epidermis overlying BCC (Fig. 2D). As



**Fig. 1.** Specificity testing of the Bax and Bcl-2 antibodies. *A)* Polyclonal antibody against Bax (Ab-1) in normal skin. Note sporadic expression in basal layer of the epidermis and more extensive expression in the suprabasal layers. *B)* Polyclonal antibody against Bax (Ab-1) pre-incubated with blocking peptide: no staining present. *C)* Polyclonal antibody against Bcl-2 (N-19) in basal cell carcinoma (BCC): epidermis and tumor cells show expression of the Bcl-2 protein. *D)* Polyclonal antibody against Bcl-2 (N-19) pre-incubated with blocking peptide: no staining present. *E)* Monoclonal antibody against Bcl-2 (clone 100) in normal skin: basal layer positive, suprabasal layers negative. *F)* Polyclonal antibody against Bcl-2 (N-19) in normal skin: comparable result as observed for the monoclonal antibody. *G)* Immunoblotting results with the monoclonal (clone 124, lane a) and polyclonal antibody (N-19, lanes b and c) against Bcl-2: a single band is detected with both antibodies at the expected molecular weight of 29 kDa in the MCF-7 and Jurkat cell lines. *H)* Immunoblot with the polyclonal antibody against Bax (Ab-1): one single band was detected at the expected molecular weight of 21 kDa in 3T3, HT29 and HeLa cell lines. (The 40 $\times$  objective was used for photography of images A-F.)



**Fig. 2.** Proliferation- and apoptosis markers in the epidermis. *A)* Bcl-2 (green) and Ki-67 (red): Bcl-2 was found to be present in the basal cell layer of the epidermis of normal skin, whereas Ki-67 is mainly restricted to the parabasal layer. *B)* Bcl-2 (green) and Ki-67 (red): there are some clear differences in expression of Bcl-2 and Ki-67 in the epidermis overlying BCC as compared with the expression patterns in normal epidermis. The expression is enhanced and simultaneous expression of the two markers occurs. *C)* Bcl-2 (green) and Bax (red): Bax shows a low level of expression in the basal cell layer but is up-regulated in the suprabasal layers of the epidermis. The expression patterns of Bcl-2 and Bax are mutually exclusive. *D)* Bcl-2 (green) and Bax (red): a clear difference between the expression patterns of Bcl-2 and Bax in epidermis overlying BCC and normal epidermis is that simultaneous expression of Bcl-2 and Bax can occur in some parabasal cells (see arrows). *E)* Bax (red) and Ki-67 (green): the expression patterns of Bax and Ki-67 can show overlap in some parabasal cells (see arrows). *F)* Bax (red) and Ki-67 (green): increased number of cells showing Ki-67 expression in the epidermis overlying a tumor as compared with normal epidermis. (The 40 $\times$  objective was used for photography of images A-F.)

in normal epidermis, the Bax and Ki-67 staining patterns in the epidermis overlying BCC occasionally showed co-expression (Fig. 2F).

### Proliferation and apoptosis markers in BCC (Table 3)

A homogeneous staining pattern was found for Bcl-2 in BCC throughout the entire lesion, for both the polyclonal and the monoclonal antibody. Expression of Ki-67 was shown in the tumor cells at the periphery of most of the nodules in the nodular type of BCC, and in a somewhat more variable pattern in the morpheaform type of BCC. The percentages of Ki-67-expressing cells varied greatly amongst the various histological types and within an individual tumor (6–32%), with an average of almost 20% (Table 3). Areas with a high number of cells expressing Ki-67 occurred, however, adjacent to areas with a lower number of Ki-67-positive cells (Fig. 3A).

The expression of the Bax protein showed a scattered staining pattern which varied among the different types of BCC and within the individual tumors (Fig. 3B), but on average it was found in approximately 80% of the tumor cells. The percentages of cells showing simultaneous expression of Bax and Ki-67 varied widely (4–25%) between tumors (Table 3 and Fig. 3C), and a correlation with the histopathological type could not be found. The proliferative (Ki-67-positive) fraction of the tumor consisted of cells either expressing or not expressing Bax. It is shown in Fig. 4 that within the proliferative compartment of each individual tumor the

percentage of Bax-positive cells was higher than the percentage of Bax-negative cells.

Based on the different percentages of cells staining positive for the three markers, we can distinguish three different tumor cell fractions (Table 4). The progressive fraction, which stains positive for Bcl-2 and Ki-67, but is negative for Bax, accounts for  $6.4 \pm 2.6\%$  of the tumor cells. This fraction of cells may be thought of as being responsible for the growth of the tumor since it is actively proliferating and protected from undergoing apoptosis. The fraction of cells that expresses all three markers may be regarded as proliferating but still prone to apoptosis because of the presence of Bax. This fraction comprises  $13.0 \pm 5.2\%$  of the tumor cells. The third fraction ( $47 \pm 6.2\%$ ), expressing Bax and Bcl-2, but lacking expression of Ki-67, is the non-proliferating fraction that is still prone to apoptosis.

### Discussion

The growth rate in neoplastic lesions is often determined by both an increase in the fraction of proliferating cells and a decrease in the rate of cell loss.<sup>14</sup> It has become apparent that in BCC an imbalance between apoptosis and proliferation may explain its progression. Candidates for regulating the rate of cell death are Bcl-2, which prevents apoptosis,<sup>15–19</sup> and

Table 3. Frequency of expression of proliferation and apoptosis markers in basal cell carcinoma (BCC)

Subtype	Patient number	Ki-67+	Bax+	Ki-67+/Bax–	Ki-67+/Bax+
Nodular	1	9%	82%	3%	4%
Nodular	2	19%	87%	7%	11%
Nodular	3	27%	85%	10%	19%
Nodular	4	26%	83%	8%	17%
Nodular	5	22%	74%	4%	16%
Nodular	6	6%	77%	2%	3%
Nodular	7	17%	82%	8%	10%
Nodular	8	24%	82%	8%	16%
Nodular	9	23%	87%	7%	16%
Nodular	10	25%	80%	9%	15%
Nodular	11	8%	70%	3%	5%
Nodular	12	27%	82%	4%	21%
Nod/morph	13	21%	87%	5%	15%
Nod/morph	14	14%	81%	4%	13%
Nod/aden	15	23%	67%	12%	12%
Nod/aden	16	23%	77%	8%	14%
Morphea	17	32%	83%	10%	20%
Mnod/morph	18	25%	60%	4%	25%
Mnod/morph	19	32%	72%	6%	16%
Mnod/morph	20	20%	78%	6%	16%
Mnod/morph	21	14%	79%	5%	9%
Mnod/morph	22	27%	80%	8%	19%
Mnod/morph	23	22%	79%	4%	19%
Micronodular	24	12%	88%	3%	10%
Micronodular	25	15%	79%	6%	8%
Micronodular	26	14%	87%	5%	8%
Mixed	27	23%	65%	11%	12%
Mixed	28	18%	76%	8%	12%
Mixed	29	12%	88%	4%	8%
Mixed	30	17%	77%	8%	16%
Mean percentage		19.7%	80.1%	6.4%	13.0%
SD		6.8%	7.0%	2.6%	5.2%

Nod/morph, nodular/morpheaform; Nod/aden, nodular/adenoid; Mnod/morph, micronodular/morpheaform; SD, standard deviation.



**Fig. 3.** Proliferation and apoptosis markers in the BCC. *A)* Bcl-2 (red) and Ki-67 (green): BCC stains homogeneously positive for Bcl-2, while Ki-67 yields positive staining at the periphery of the nodules. Morphic strands show a more scattered pattern of Ki-67 expression. *B)* Bcl-2 (green) and Bax (red): BCC expresses Bcl-2 homogeneously, while Bax expression is found in 60–90% of the tumor cells. *C)* Bax (red) and Ki-67 (green): simultaneous expression occurs in tumor cells (white arrows), while tumor cells are also positive for Ki-67 but negative for Bax (yellow arrows). (The 40× objective was used for photography of images A–C.)

process of apoptosis is induced or suppressed.<sup>10</sup> To detect cell proliferation, Ki-67 antibodies are most widely used. They bind to a nuclear antigen expressed by cycling cells.<sup>21,22</sup>

Our results in normal skin are in concert with previously described studies, showing Bcl-2 expression in the basal layer, Ki-67 expression in the parabasal layer and Bax staining in a few basal cells, but predominantly in the suprabasal layers.<sup>23,24</sup> Our double-label immunoassays clearly demonstrated the almost exclusive staining patterns for Ki-67 and Bcl-2 on the one hand, and for Bcl-2 and Bax on the other. A few cells, which are most likely responsible for the regeneration of the epidermis, expressed both Ki-67 and Bcl-2. The expression of Bax in the suprabasal compartment may reflect the commitment of these cells to undergo terminal differentiation and eventually apoptosis, which also explains the down-regulation of Bcl-2 in these cells.

The expression of these markers in the epidermis overlying BCC has not been studied extensively before. Our results showed Bax expression comparable with that in normal epidermis, but expanded staining with the polyclonal Bcl-2 antibody in the epidermis overlying BCC. The differences in staining patterns obtained by the monoclonal and polyclonal Bcl-2 antibodies may be explained by the recognition of different epitopes. The epitope recognized by the monoclonal Bcl-2 antibody is apparently shielded in the suprabasal cell layers by heterodimerization of Bcl-2 with other proteins, e.g. Bax. Similar observations of epitope shielding have been described by Schandl et al.<sup>25</sup>

The fraction of parabasal cells staining for Ki-67 was increased in the epidermis overlying BCC as compared with that in the epidermis of normal skin, while also more suprabasally located cells were Ki-67-positive. This suggests loss of cell cycle control and maintenance of the Bcl-2/Bax status in the process of migration of basal cells towards the suprabasal layer in the epidermis overlying BCC. A significantly increased Ki-67 expression was also detected in the hyperplastic epidermis overlying a keratotic melanocytic nevus, whereas Bcl-2 and Bax were expressed

Bax, which is known to induce apoptosis.<sup>18,20</sup> These two proteins can form homodimers and heterodimers, and their balance eventually determines whether the

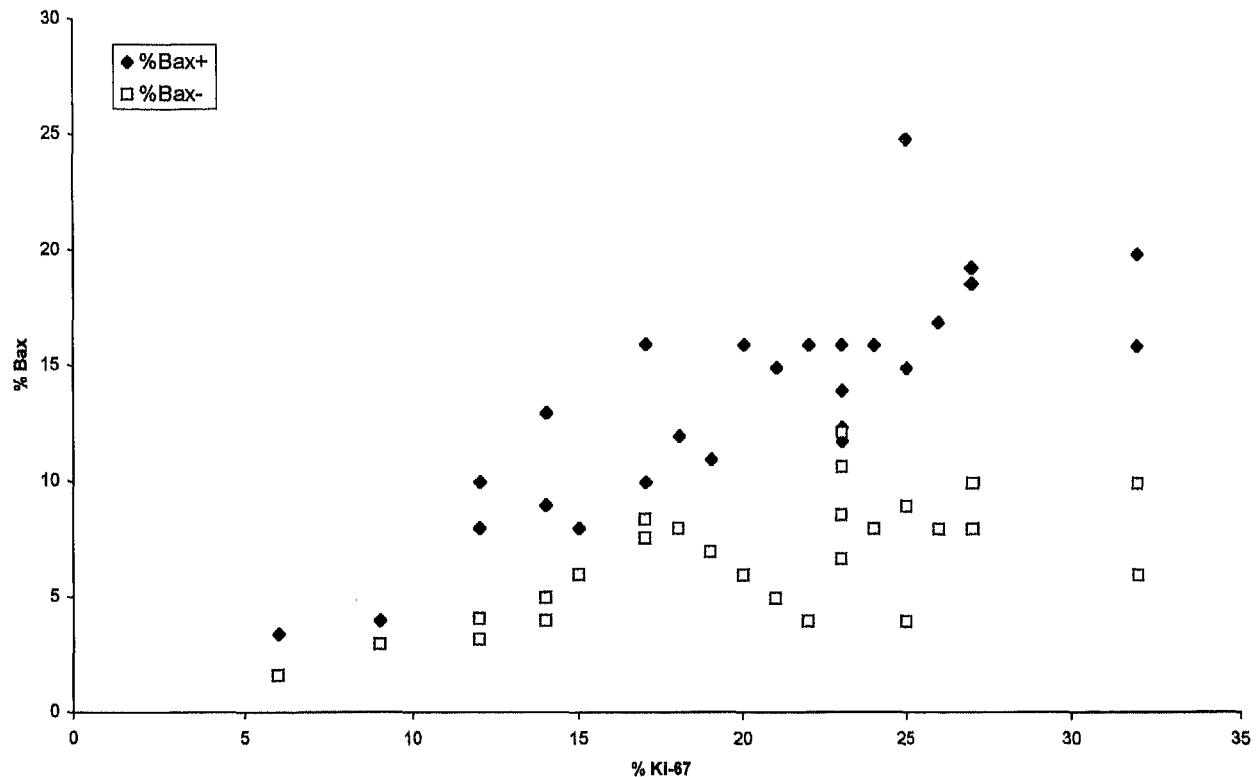


Fig. 4. Bax status of the proliferative compartment. The graph represents the percentage of Bax-positive and -negative cells within the Ki-67 positive fraction. From this graph it can be seen that, in the proliferative compartment of each individual tumor, the percentage of Bax-positive cells is higher than the percentage of Bax-negative cells.

similarly in both normal and hyperplastic epidermis.<sup>26</sup> This demonstrates increased proliferation in the context of adequately regulated apoptosis.

A similar mechanism may explain the phenotype of BCC, in which a cell fraction occurs which expresses both Bcl-2 and Ki-67, thus representing the actively proliferating fraction. The percentage of these cells, which are double positive for Ki-67 and Bcl-2, varied widely between tumors (6–32%) and also within the individual tumors. A correlation between the histological type of the tumor and the percentage of Ki-67-positive cells could not be found. Nevertheless, it may be obvious that these double-positive cells play a key role in the progression of the tumor, one reason being that they are prone to accumulation of genetic modifications.

A comparison between the growth behavior of trichoepithelioma (TE), a benign cutaneous tumor that originates from hair follicles,<sup>27</sup> and BCC shows that BCC and TE exhibit differences in both Ki-67 and Bcl-2 expression.<sup>28,29</sup> TE is a tumor characterized by proliferation of keratinocytes that rapidly differentiate into mature follicular cells, and which apparently cease to express Bcl-2. BCC is composed of a population of basaloid keratinocytes, which largely retain their ability to produce Bcl-2.<sup>29</sup> Furthermore, the number of Ki-67-expressing cells is significantly

higher in BCC,<sup>28</sup> which may explain the differences in growth behavior between these tumors. The consistent lack of expression of Bcl-2 in squamous cell carcinoma (SCC), a common malignant neoplasm that arises in the skin, contrasts with the staining seen in BCCs and suggests that these malignancies do not share the same cells of origin. Furthermore, their difference in expression of Bcl-2 reflects their difference in regulation of cell turnover.<sup>30</sup>

In BCC cells, a variable Bax expression pattern was found. The relatively large fraction of the cells expressing Bax in the tumor seems rather unexpected because this protein is known to act as a tumor suppressor by enabling cell loss through apoptosis.<sup>10</sup> Previous studies on the expression of Bax in BCC described conflicting results. Rossen et al.<sup>31</sup> showed that

Table 4. Frequency of different cell populations in basal cell carcinoma (BCC)

Fraction	Marker pattern	Percentage of tumour cells
Progressive Proliferative,	Bcl-2+/Ki-67+/Bax-	6.4 ± 2.6
prone to apoptosis	Bcl-2+/Ki-67+/Bax+	13.0 ± 5.2
Non-proliferative, prone to apoptosis	Bcl-2+/Ki-67-/Bax+	47.6 ± 6.2



the protein is not expressed in BCC, whereas Delehedde et al.<sup>23</sup> demonstrated that the Bax protein is in fact expressed in BCC. We found an expression pattern similar to that of Delehedde et al.<sup>23</sup>, using the same antibody as used by Rossen et al.<sup>25</sup> However, we used frozen tissue sections, while these authors used paraffin-embedded material, which may explain the different results.

It was shown in previous studies<sup>6,32,33</sup> that, based on morphology, the frequency of apoptotic cells in different types of BCC ranged from 0.6 to 51%. This corroborates our findings that approximately 80% of the BCC cells express Bax and is therefore prone to apoptosis. This relatively high percentage of Bax-expressing cells, combined with the relatively low fraction of the cells expressing both Ki-67 and Bcl-2, may explain the slow progression rate of BCC.

In line with our finding that a relatively large proportion of the BCC cells express the Bax protein, Mooney et al.<sup>6</sup> described that the apoptosis:mitosis ratio for BCC was found to be 15.1, as compared with 2.1 for melanoma. The finding by several authors that up to 50% of the BCC cells can be apoptotic<sup>6,26,27</sup> is now explained by the high percentages of cells expressing Bax. The question remains whether Bcl-2 is still functional. Previous studies already mentioned Bcl-2 mutations. Huang et al.<sup>34</sup> showed that, when the serine residue at position 70 of the Bcl-2 protein is altered into an alanine residue, the anti-apoptosis function of the protein is diminished because the protein cannot be dephosphorylated. Other inactivating mutations were deletion of the conserved BH4 domain, as well as G145E and W188A mutations.<sup>34</sup> Furthermore, Uhlmann et al.<sup>35</sup> showed that the deletion of a non-conserved region between residues 51 and 85 of human Bcl-2 resulted in an enhanced interaction with the death-promoting protein Bax, but no correlation with the ability of Bcl-2 to interact with other proteins was observed.

In summary we can state that the results of our study show a relatively small progressive cell fraction in BCC which is protected from apoptosis and actively proliferating. The failure of these cancer cells to undergo programmed cell death may contribute substantially to tumor progression. It has, however, also become clear that a relatively high percentage of BCC cells express considerable levels of Bax, a pro-apoptotic protein. In particular, the presence of this component in BCC may explain its indolent growth behavior.

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